METHODOLOGIES FOR PRODUCING AMYLOSE: A REVIEW

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Abstract

Three main *in vitro* approaches can be distinguished for obtaining amylose (AM): enzymatic synthesis, AM leaching, and AM complexation following starch dispersion. The first uses α-D-glucose-1-phosphate (G1P), a glucosyl primer with a degree of polymerization (DP) of at least 4 and phosphorylase (EC 2.4.1.1), commonly from potatoes. Such approach provides AM chains with low polydispersity, the average DP of which can be manipulated by varying the reaction time and the ratio between G1P, primer and enzyme dose. AM leaching is the result of heating a starch suspension above the gelatinization temperature. This approach allows isolating AM on large scale. The AM DP, yield, and purity depend on the heating rate, leaching temperature, shear forces and botanical origin. High leaching temperatures (80-85˚C) result in mostly pure AM of DP >1000. At higher temperatures, lower purity AM is obtained due to amylopectin leaching. Annealing as pre-treatment and ultracentrifugation or repetitive organic solvent based precipitations after leaching are strategies which improve the purity of AM extracts. When AM is separated by complex formation, complete dispersion of starch is followed by bringing AM into contact with *e.g. n*-butanol or thymol. The resultant complex is separated from amylopectin as a precipitate. Complete starch dispersion without degradation is critical for obtaining AM of high purity. Finally, higher DP AM can be converted enzymatically into AM fractions of lower DP.

Keywords: Amylose, Enzymatic Synthesis, Starch Fractionation, Aqueous Leaching, Complex Formation

1. Introduction

Starch has a wide range of applications in food and non-food based industries. It is the main reserve polysaccharide of cereal grains (*e.g.*, wheat, rice, maize, etc.), roots, and tubers (*e.g.*, potato, cassava, etc.) (Schwartz & Whistler, 2009; Delcour & Hoseney, 2010) and a main energy source in the human diet (Jéquier, 1994). Normal starch granules contain two types of glucose polymers: highly branched amylopectin (AP) and almost linear amylose (AM) [commonly ~70-82% and ~18-30% by weight, respectively (Tester et al., 2004)]. These polymers differ in molecular structure (Hirst et al., 1972; Mua & Jackson, 1997a), architectural arrangement within the granule (Takeda et al., 1987; Jane et al., 1992; Kasemsuwan & Jane, 1994; Saibene & Seetharaman, 2010), physicochemical properties and functionality (Mua & Jackson, 1997b; Wang et al., 1998; Singh et al., 2010).

As a result of the growing interest in its functional properties, AM has become the focus of different studies (AAF, 2013). Native high AM maize starch granules resist human amylolytic digestion and are referred to as resistant starch type 2 (Topping et al., 2003) and a source of dietary fiber with beneficial health effects (Bird et al., 2009; Martinez et al., 2010; Keenan et al., 2013), as is resistant starch type 3 (*i.e.*, retrograded starch) (Eerlingen & Delcour, 1995; Topping et al., 2003; Bird et al., 2010), the enzyme resistance of which is the result of it containing crystalline AM. Resistant starches are used in some food applications (Fuentes-Zaragoza et al., 2010).

In diverse food systems such as baked goods, AM forms complexes with some lipids (Putseys et al., 2010). In breadmaking, AM lipid complex formation lowers initial bread firmness (Pareyt et al., 2011). AM also has potential in wrapping chemistry (Numata & Shinkai, 2011) and it can

form spherulites (Helbert et al., 1993; Ma et al., 2011) and fibers (Morishita et al., 2005; Liu & Han, 2006) with good mechanical properties. Moreover, it has been used in targeted drug delivery studies as carrier molecule (Milojevic et al., 1995), for trapping flavors (*e.g.*, hexanal, hexanone, fenchone, …) (Wulff et al., 2005; Jouquand et al., 2006), and synthesis of films and foams (Lourdin et al., 1995). In some of the mentioned applications, the molecular weight (MW) distribution of AM plays an important role (Mua & Jackson, 1997b).

In many studies, deductions have been made about the properties of AM from its behavior in starch systems which evidently also contain AP. From this point of view, it is useful to prepare pure AM for studies aiming to fully understand its functionality in different applications. This review discusses the current methodologies for producing AM with a focus on the properties of the obtained AM. Current commercial AMs have wide molecular size distribution and average degrees of polymerization (DP). This review will serve as a tool to assess and adapt available methodologies for preparing AM with varying MW distribution and purity.

2. Amylose: a general overview

In Nature, starch occurs as granules with different morphologies (*e.g.* spherical/rounded, lenticular, and polygonal) depending on the botanical source and composition (van de Velde et al., 2002; Tester et al., 2004; Delcour et al., 2010; Waterschoot et al., 2014). The granules consist of alternating semi-crystalline and amorphous growth rings. AM and the branching points of AP are amorphous, whereas the double helices of AP are crystalline (Wang et al., 1998; Tester et al., 2004). Starch is thus a semi-crystalline system. Three types of X-ray diffraction patterns can be distinguished depending on the botanical source: A-type (cereal starches), B-type (tuber starches, high AM starches and retrograded starches) and C-type

(legumes and beans) (Wang et al., 1998). While in the starch granule AM is amorphous, it can form crystalline structures with A- (Hsien-Chih & Sarko, 1978), B- (Hsien-Chih & Sarko, 1978), and C-type (Sarko & Wu, 1978) X-ray diffraction patterns. After melting of AP crystals during gelatinization in excess water, during subsequent cooling, AM crystallizes as B-type crystals on a short term (Miles et al., 1985; Goesaert et al., 2005).

AM crystals are the result of the tendency of AM to gain stability by forming double helices when not complexed with suitable hydrophobic molecules. The A-, B-, and C- types of AM crystals have different structural building units and hence vary in architecture (Table I). A-type AM has a monoclinic unit cell (a = 2.124 nm, b = 1.172 nm, c = 1.069 nm) with left-handed double helices packed in a pitch height of 2.08-2.38 nm and can hold eight water molecules (Imberty et al., 1988; Imberty et al., 1991). B-type unit cells are of hexagonal geometry ($a = b$ = 1.85 nm, c = 1.04 nm) with six-fold left-handed double-helices with 36 water molecules and the same pitch height as A-type AM (Imberty & Perez, 1988; Imberty et al., 1991). The C-type is considered to be a mixture between A- and B- type crystals (Cairns et al., 1997); crystal dimensions and packing density depend on the proportions of the A- and B- polymorphs (Gernat et al., 1990; Gernat et al., 1993; Cairns et al., 1997). Water in A-type AM crystals is present in discrete pockets formed by a very tight network of hydrogen bonds, whereas in B-type crystals it is located in channels between the double helices (Popov et al., 2009). This presumably causes differences in thermal properties of the polymorphs. A-type AM melts at temperatures about 20 ˚C higher than B-type AM at water contents exceeding 40% (Whittam et al., 1990). Evidently, crystal perfection can affect the thermal behavior. Also, the crystal properties of AM may be affected by the MW of the polymer. To the best of our knowledge, little information is available on this.

While AP is considered to be one of the largest polymers in Nature, AM is a smaller almost linear molecule (Tester et al., 2004). The vast majority of glucose units in AM are linked by α-(1→4) bonds with less than 1% α-(1→6) glucosidic bonds (Hizukuri et al., 1981; Curá et al., 1995). The weight-average MW of AM varies considerably with the botanical source and ranges from $1x10^5$ – $9x10^6$ (Ong et al., 1994; Buléon et al., 1998), corresponding to a DP of about $5x10^2$ – $6x10^3$ (Table II). In contrast, the weight-average MW of AP reaches values up to $1x10^9$ (Tester et al., 2004).

AM plays an important role in starch gelation (Copeland et al., 2009), but insight in its precise role has been obscured by the presence of AP. Recent studies have indicated the formation of fractal objects in the form of blurred cylinders during cooling of gelatinized starch suspensions in the absence of shear (Putseys et al., 2011). Moreover, during storage of such suspensions, AM chains gain crystallinity after a few hours and are then the main responsible for the initial firmness of a starch gel (Miles et al., 1985). However, the specific influence of AM MW on formation and dimension of the fractal objects has, to the best of our knowledge, not been studied.

In the presence of suitable ligands, AM can form single left-handed helices when in complex with the (non-polar) moieties of *e.g.* ethanol, *n*-butanol, isopropanol, fatty acids, and dimethyl-sulfoxide (Le Bail et al., 2005; Putseys et al., 2010). The cavity in the interior of the helix is hydrophobic, whereas the outer surface is hydrophilic (Gessler et al., 1999; Nimz et al., 2004). When complexed, such single helices are referred to as AM-inclusion complexes and can either remain amorphous (Type I) or organize themselves into crystals (Type II) (Putseys et al., 2010).

The latter are referred as V-type crystals (Welland & Donald, 1991; Wulff & Kubik, 1992). They can give rise to lamellar assemblies with amorphous regions at the folding sites of the molecule (Zobel et al., 1967; Biliaderis et al., 1986; Eerlingen et al., 1993). These lamellae can further organize into larger crystals with either a platelet (Jane & Robyt, 1984; Whittam et al., 1989) or a spherical shape (Shogren et al., 2006; Singh et al., 2010; Ma et al., 2011). The dimensions and properties of the V-type unit cell of AM inclusion complexes depend on the ligand (Nuessli et al., 2003; Le Bail et al., 2005; Putseys et al., 2010).

3. Tailor made amylose chains via an upstream procedure

In an effort to mimic the ability of Nature to synthesize glucose polymers, AM has been synthesized enzymatically. Low DP AM ($10 < DP < 20$) can be synthesized by transferring monomers from cyclomaltohexaose to α-malto-oligosaccharides ($DP \geq 7$) via cyclomaltodextrin glucanotransferase (EC 2.4.1.19) (Niemann et al., 1992). However, enzymatic polymerization using phosphorylases (EC 2.4.1.1) is a more common protocol. Such *in vitro* procedure has been introduced by Cori and Cori (1940) who suggested to use maltoheptaose as primer and glucose-1-phosphate (G1P) as source of glucose units to be transferred to the non-reducing end of the chain by phosphorylase. The reaction releases inorganic phosphate. Potato phosphorylase (with optimal activity at pH 6.2 and 37˚C) is commonly used in synthesis of AM chains as it is easily recovered by ammonium sulfate precipitation (Roger et al., 2000; Morishita et al., 2005). One of the disadvantages of this procedure is the cost of the substrate (G1P). Fortunately, G1P can be synthesized from glucose using sucrose phosphorylase (EC 2.4.1.7). In the coupled reaction with potato phosphorylase, sucrose is utilized to produce G1P and phosphate is recycled during the procedure (Figure 1). The resulting AM has low polydispersity (Waldmann et al., 1986).

Phosphorylase catalyzed polymerization allows producing tailor made AM chains because the size of AM is controlled directly by the G1P/primer ratio and incubation time (Fujii et al., 2003). Thus, it is essential to take into account the stoichiometry of the reaction. The yield in the polymerization reaction ranges from 60% (Praznik & Ebermann, 1979) to 90% (Waldmann et al., 1986). Even though the conversion yield is high, the reaction has been applied in exploratory and small scale laboratory studies. For the same level of primer, the average DP is related to the dose of G1P, whereas a direct relation between incubation time and average DP is observed when maintaining the doses of G1P and primer constant (Roger et al., 2000).

The reaction can be stopped by heating to above 90 ˚C (Waldmann et al., 1986) or by centrifugation in which precipitated AM is separated from the reaction medium (Putseys et al., 2009). Another approach is to add a complex forming agent such as *n*-butanol which gives rise to an insoluble clathrate (Putseys et al., 2010). In the presence of a hydrophobic guest polymer, AM chains of sufficient length form complexes which precipitate (Gelders et al., 2005). In a different mechanism known as vine-twinning polymerization (which, as its name implies, resembles the growth of vine plants twinning around a rod), AM chains are enzymatically synthesized around the guest and then give rise to the complex (Kadokawa et al., 2003; Kaneko & Kadokawa, 2005; Kadokawa & Kobayashi, 2010). An intermediate mechanism has been put forward. It suggests that the synthesis of an AM molecule long enough to bind the guest is needed before complex formation takes place. This would be followed by continuous extension of the chain via vine-twinning until the complex becomes insoluble and thus precipitates (Putseys et al., 2009).

Whatever be the case, AM-lipid complexes can be synthesized using potato phosphorylase during in situ synthesis of AM (Gelders et al., 2005; Putseys et al., 2009; Putseys et al., 2010).

4. Aqueous leaching of amylose: harvesting diffused molecules

When a starch-water system is heated in excess water, starch granules start swelling (Ratnayake & Jackson, 2008). When the system reaches the gelatinization temperature, the granules lose their birefringence, continue to swell and finally disrupt (Waigh et al., 2000; Delcour & Hoseney, 2010). The AM chains start to leach from the granule and the viscosity of the dispersion changes (Waigh et al., 2000; Goesaert et al., 2005; Vermeylen et al., 2006; Gomand et al., 2010; Doutch et al., 2012). The art of aqueous leaching of AM consists of doing it in a way which avoids starch granule disruption and AP solubilisation but still solubilizes AM (Adkins & Greenwood, 1966; Shi et al., 1991; Vorwerg et al., 2002). Leaching followed by centrifugation produces a gel phase containing granule remnants enriched in AP and an aqueous phase containing mainly leached AM (Tester & Morrison, 1990; Hermansson & Svegmark, 1996; Waigh et al., 2000) (Figure 2). When one considers AM leaching as a diffusion based mass transfer, its exudation from the starch matrix can be influenced by the rate of diffusion of water into the solid (*e.g.*, when more water is able to enter the granules, the AM chains in the amorphous zone may gain more mobility and then leach out easier) (Sakonidou et al., 2003; Russo et al., 2007).

Several factors influence leaching of AM during heating. For one, the extraction yield is influenced by the starch concentration. In fact, 2-3% wheat and maize starch dispersions provided

196 higher yields of extracted AM (*i.e.* 18-21%, starch basis), whereas starch concentrations between
197 0.5 – 1.5% or between 4.0 – 6.0% resulted in 20 – 50% less extracted AM (Shi et al., 1991).
198 However, heating temperature and rate have a more drastic effect on aqueous leaching than the
199 initial concentration of the dispersion (Radosta et al., 1992; Jacobs et al., 1995). The average MW
200 of recovered AM increases with the leaching temperature for wheat (Ghiasi et al., 1982; Shi et
201 al., 1991), maize (Shi et al., 1991; Roger & Colonna, 1996), and rye (Radosta et al., 1992)
202 starches. The yield of leached product can also be increased by increasing the temperature, but
203 this is at the expense of the purity of the extract (Vorwerg et al., 2002). Shi and co-workers
204 (1991) showed that leaching of wheat and maize starches at 95 ˚C provides polymers of higher
205 DP (>1000) than treatment at 75 ˚C (DP ~800). Lourdin and co-workers (1995) leached AM from
206 pea starch at 70 °C and obtained AM with an average MW of 4×10^5. In maize starch, the
207 average chain length of the obtained AM increases when the leaching temperature increases from
208 65 to 95 ˚C. However, at 85 ˚C, a marked transition from mono- to bimodal MW distributions
209 occurs with a prominent population of high MW AM ($7.0 \times 10^5 – 3.0 \times 10^6$) and a less abundant
210 lower MW AM population ($1.6 \times 10^5 – 7.3 \times 10^5$) (Roger & Colonna, 1996). The bimodal
211 distributions found by Roger and Colonna (1996) in leachates from maize starch provide
212 evidence for the heterogeneity of AM in terms of DP. More polydisperse maize AM is obtained
213 when leaching at temperatures exceeding 85°C than at lower temperature (Roger & Colonna,
214 1996). In general, polydispersity of the AM extract from maize starch is already quite high at
215 temperatures below 85 ˚C as revealed by high performance size exclusion chromatography
216 (Takeda et al., 1990; Takeda et al., 1992; Fishman et al., 1995; Roger & Colonna, 1996). The
217 temperature, at which polydispersity trends to increase on the AM extract, can be expected to
218 depend on the botanical origin of starch as swelling and gelatinization depend on it (Singh et al.,
219 2003; Gomand et al., 2010).

The extraction yield of AM during aqueous leaching can be influenced by the heating rate (Shi et al., 1991). When it is high (*i.e.*, 10 ˚C/min) and the starch is heated in excess water to 96 ˚C, after centrifugation the AP fraction remains in the sediment (Doublier, 1981), and the AM dispersed in the supernatant can be recovered. At a slow heating rate (1 ˚C/min, up to 95 ˚C, 3% wheat starch dispersion), higher yields of AM (~21-23%) are obtained than when heating at 10 °C/min (~20%). This difference is more noticeable at higher initial concentrations of starch (*i.e.*, 4.5% wheat starch), where a heating rate of 1 ˚C/min increased the extracted AM a twofold compared to a heating rate of 10 ˚C/min (from ~8% to ~16%) (Shi et al., 1991). Next, AM can be recovered from the aqueous phase by complexation with *n*-butanol (*cf. infra*). In native and annealed potato, pea and rice starches, AM leaching was positively correlated to time and temperatures up to 95 ˚C (Jacobs et al., 1995). This might be related to an increase in swelling power at higher temperatures that provides more mobility in the amorphous zones of the granules.

5. Starch fractionation via complex formation

Fractionation of starch into its two main constituents can be achieved by selective complex formation after complete dispersion followed by precipitation. The immiscibility of AM and AP follows from their thermodynamic properties and phase diagrams with water (Kalichevsky & Ring, 1987; Conde-Petit et al., 1998; Rodríguez & González de la Cruz, 2003). When starch is dispersed in water or dimethyl sulfoxide (DMSO) (Killion & Foster, 1960), AM can be separated from AP by complex forming agents (Takeda et al., 1986; Takeda & Susumu, 1987; Charoenkul et al., 2006; Naguleswaran et al., 2013). In such process, prior to complex

formation, the dispersion of AM needs to be effective if one is to obtain pure AM. Rather polydisperse AM of high MW and little contamination with AP can be obtained using this approach (Takeda et al., 1990) (Table III).

5.1 Dispersion, a key step in starch fractionation

There are different approaches to disperse starch. DMSO (Millard et al., 1997; Han & Lim, 2004) or alkaline solutions (Roger & Colonna, 1996) are commonly used. Starch is boiled in 90% DMSO for 60 min followed by stirring at room temperature during 8h to achieve optimal solubility; however, excessive boiling (≥ 2h) causes AP thermal degradation (Han & Lim, 2004). Alkaline solutions can partly degrade AM and reduce its DP (Wang & Zopf, 1989; Morishita et al., 2005). When adding urea to alkaline solutions, dissolution is enhanced and degradation reduced (Grant et al., 2002). High temperatures (*e.g.* above 120 °C) and pressurization can be used to disperse maize starch and extract polydisperse high MW AM (You & Lim, 2000). The way of obtaining a homogeneous dispersion can affect MW distribution, as very high temperatures and autoclaving can promote hydrolysis and AM agglomeration into crystalline structures (Doublier et al., 1992) and sonication can promote starch degradation (You & Lim, 2000).

5.2 Amylose complexes as simple helices

Once complete dispersion has been achieved, AP can be removed by concanavalin A. This lectin binds to AP (Matheson & Welsh, 1988; Yun & Matheson, 1990) but not to AM. It has four binding sites able to bind α-D-mannosyl and α-D-glucosyl residues at various non-reducing ends and precipitates AP (Sumner et al., 1938; Gibson et al., 1997). AM can be precipitated

from aqueous solution by complexation with some alcohols (Klucinec & Thompson, 1998; Le Bail et al., 2005; Kim et al., 2009). It forms an insoluble complex with *n*-butanol and thymol (Banks & Greenwood, 1967; Hu et al., 2013). *n*-Butanol has been extensively used to precipitate AM (Schoch, 1942; Nuessli et al., 2003; Le Bail et al., 2005; Kim et al., 2009). Octalone has also been used for separating AM (Stepanenko & Avakian, 1973) but is less selective than *n*-butanol. When using complexing agents, it is critical to effectively remove them afterwards (Hu et al., 2013). This can be done by washing with diethyl ether and ethanol and/or solvent evaporation (Mukerjea & Robyt, 2010).

Complex formation of AM with some agents leads to clear differences in solubility between the resultant AM inclusion complex and AP. The mechanisms of precipitation by thymol or *n*-butanol are similar (Kawada & Marchessault, 2004).When the solution is saturated with *n*-butanol, AM forms single helix inclusion complexes that organize into a fringed lamellar structure (Whittam et al., 1989; Curá & Krisman, 1990; Eerlingen et al., 1993; Hu et al., 2013). Such complexes result in a typical V-type X-ray diffraction pattern characterized by larger orthombic cells (Helbert & Chanzy, 1994) than those of hydrated V-type AM (Rappenecker & Zugenmaier, 1981) but smaller than those formed with isopropanol (Buléon et al., 1990; Nuessli et al., 2003; Rondeau-Mouro et al., 2004).

6. Enzymatic hydrolysis as a strategy to change the DP of amylose

Starting from AM of large MW, AM chains tailor made in terms of average DP can be produced (Eerlingen et al., 1993; Andersson et al., 2002; Gelders et al., 2004) via hydrolysis with amylolytic enzymes (Williamson et al., 1992; Eerlingen et al., 1993; Naguleswaran et al.,

289 2013). β-Amylase reduces the DP of AM to different extents depending on the incubation time
290 and enzyme dose (Eerlingen et al., 1993). A combination of α-amylase and amyloglucosidase
291 can produce AM of relatively low MW (DP<100) starting from retrograded high-AM maize
292 starch (Andersson et al., 2002). Linear dextrins (AMs) can be produced from starch by
293 liquefaction with thermostable α-amylase and subsequent saccharification with
294 amyloglucosidase (León et al., 1997; Atichokudomchai et al., 2006). The average DP of the
295 obtained material ranges from 100 down to DP 60 or even lower depending on the starting
296 material and incubation conditions (Andersson et al., 2002). Debranching enzymes such as
297 isoamylase and pullulanase produce linear chains of low average DP (Hizukuri et al., 1981).
298 Furthermore, as AP undergoes hydrolysis at the branching points by isoamylase and pullulanase,
299 the polydispersity of the final product highly depends on the degree of branching of AP
300 (Naguleswaran et al., 2013).

301

302 In water, AM readily forms fibrous structures (Shogren, 2007) which can be oriented into
303 ordered crystalline structures (Montensati et al., 2010). Differences in crystalline structures
304 result from differences in temperature, initial AM concentration and its average DP. High
305 concentrations of isolated AM (i.e., 50% aqueous dispersion, 30˚C) promote an A-type structure,
306 whereas lower values (i.e., 30% aqueous dispersion, 15˚C) provide B- or C-type crystals (Gidley
307 & Bulpin, 1987) that, as described in a previous section, are less stable and can shift to an A-
308 type polymorph (Gidley & Bulpin, 1987; Pfannemüller, 1987; Popov et al., 2009; Montesanti et
309 al., 2010). The minimum chain length of glucose polymers needed to form double helices and
310 crystallise is 10 glucosyl units. Low DPs (9-12) correspond to less than two turns of the helix
311 and promote A-type crystal formation (Gidley & Bulpin, 1987; Popov et al., 2009). The

formation of B-type crystals is favored by longer chains (DP>13) (Gidley & Bulpin, 1987; Pfannemüller, 1987). Moreover, not just the type of crystal but also its size is influenced by the DP of AM. AM chains with DPs ranging between 10-20 can arrange themselves into large crystals (5-10 μm), whereas longer chains (DP>40) lead to a weak crystalline network characterized by small crystals (<5 μm) (Montensanti et al., 2010).

7. Strategies to improve purity of amylose extracts

Annealing of starch [*i.e.*, hydrothermal treatment of a starch suspension in excess water between the glass transition and gelatinization temperatures (Jacobs & Delcour, 1998)] prior to leaching increases the purity but not the yield of AM (Zavareze & Dias, 2011). This has to be further explored as the structural modifications and mechanisms leading to higher AM purity are not fully understood. Ultracentrifugation has also been considered as a way to purify AM extracts (Majzoobi et al., 2003) as has been repetitive precipitation after successive complex formation steps (Corcuera et al., 2007). A combination of different technologies can be more effective at improving the purity and yield of the extracts. By combining leaching with selective complex formation with *n*-butanol and ultracentrifugation, one can obtain AM extracts of high purity and low polydispersity.

Jacobs and co-workers (1995) found that annealing of wheat but not of pea or potato starch tends to slightly increase leaching of AM at 95 °C. However, one can logically assume that at high temperatures (~ 95-100 °C) AP can also be leached (Ghiasi et al., 1982). Indeed, at 95 °C, AP leaching may also occur as observed by Shi and co-workers (1991) for maize and wheat starches. Thus, the increased AM leaching from rye (Radosta et al., 1992) and maize (Roger & Colonna, 1996) starches may to a degree be attributed to AP contamination. That lower leaching

temperatures (e.g. 50 °C) result in high AM purities has also been related to the effect of annealing. It has indeed been suggested that low heating rates (1˚C/min) up to high temperatures may induce an effect on wheat and maize starch granules similar to that during annealing (Shi et al., 1991). The annealing process does not affect the crystalline structure of the starch granule (Gomand et al., 2012). However, the swelling power as well as AM leaching of annealed starch are in general lower than those of native starch (Tester & Debon, 2000; Chung et al., 2009). Chung and co-workers (2009) found that annealing lowers the swelling power and AM leaching at 80 °C of pea and lentil but not of maize starches. They attributed this to the higher level (>35%) of AM in pea and lentil starches. Less AM may be leached from annealed starches due to an increased stability of the crystalline lamellae in general and, more in particular, an increase in crystal thickness or surface free energy (Gomand et al., 2012). Annealing may also be accompanied by increased crystallite perfection which itself is related to a significant decrease in granule hydration (Zavareze & Dias, 2011). The increased stability of AP double helices and the greater mobility in the amorphous zones after annealing may affect AM leaching to such extent that less but more pure AM leaches out.

Roger and Colonna (1996) observed that ultracentrifugation (15 h, 200,000 g, 10 ˚C) increases the purity of AM extracts from maize starch. The effectiveness of the treatment (*i.e.*, changes in weight-average MW and polydispersity before and after ultracentrifugation) was assessed with size-exclusion chromatography with multi-angle laser light scattering. After ultracentrifugation, AM leached at temperatures between 65-95 ˚C had reduced polydispersity and lower MW than that for extracts where ultracentrifugation was not implemented (Roger et al., 1996). The presence of AP in the leachate product at temperatures higher than 85 ˚C was

confirmed by a decrease in the iodine binding capacity of the extract after ultracentrifugation (from 21.4mg/100mg at 85 ˚C to 17.7mg/100mg at 95 ˚C (Roger & Colonna, 1996)). Thus, in the particular case of maize starch leaching should be done at temperatures below 85 ˚C to avoid AP contamination. In the case of complex formation with *n*-butanol, ultracentrifugation has been a crucial and effective treatment to remove AP contamination (Takeda et al., 1986). Majzoobi and co-workers (2003), when using ultracentrifugation as a procedure for fractionating dispersed wheat starch in DMSO, were able to obtain highly pure AP but not highly pure AM.

Finally, it is of note that starch and its derivatives are susceptible to air oxidation (Bala-Piasek & Tomasik, 1999). Thus, storage time and conditions also can influence the properties of AM (Gilbert, 1958). Oxidation takes place in the amorphous zones of the starch granule (where AM is present) (Kuakpetoon & Wang, 2001). However, to the best of our knowledge, the specific mechanism is not yet well known.

8. Conclusions

In enzymatic synthesis of AM, AM properties are tailored by varying the ratios between substrate and primer and incubation time. This method provides AM of high purity.

Fractionation of starch into its two main constituents can be achieved by aqueous leaching or selective complex formation after dispersion of starch followed by precipitation. Both of these methods provide AM extracts that differ in purity, MW, and extraction yield depending on the procedure.

Leaching procedures are combined with phase separation. However, the leaching temperature must be carefully chosen to limit the degree of polydispersity and to avoid contamination with

AP. Complex formation as an additional purification process is recommended to circumvent AP contamination when leaching at high temperatures. Moreover, annealing prior to leaching increases the purity of the AM extract as leaching becomes more selective due to the crystal stability within the granule. Once amylose of high MW is produced, its DP can be further manipulated by using enzymes.

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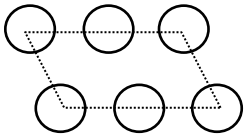
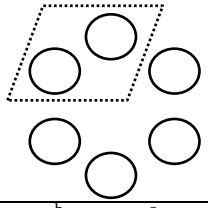
762

763 **Table I.** The crystalline structure of amylose polymorphs[1]

	Amylose crystalline type		
	A-Type	**B-Type**	**C-Type**
Unit cell [d, e, f]	Monoclinic	Hexagonal	A and B-type hybrid
Pitch (Å) [c, f]	10.52	10.45	10.47
Chirality [d, e, f]	Left handed	Left handed	--
Helix type [d, e]	Double Parallel stranded Parallel packed	Double Parallel stranded Parallel packed	--
Water [a, b, c, f]	8 molecules per cell	36 molecules per cell	--
Density [c]	1.56	1.4	--
Arrangement [d, e, f, g]			--

764 [1]Values previously reported by: [a] Hsien-Chih, & Sarko, 1978a, [b] 1978b; [c] Sarko & Wu, 1978; [d] Imberty et al., 1988; [e]

765 Imberty & Perez, 1988; [f] Buléon et al., 1998; [g] Putseys, et al., 2011

766 **Table II.** Properties of amylose from different botanical origins[1,2]

	Property			
Source	Iodine binding capacity (g 100 g^{-1})	DP_w (range) [a]	DP_n	Polydispersity [a]
Wheat	19.9 [a]	--	1290 [a]	--
Maize	20.0 [a] - 21.1 [b]	390 – 13100	960 [a]	2.66
Rice	20.0 [a] - 21.1 [a]	210 – 12900	920 [a] – 1110 [a]	2.64 - 3.39
Potato	19.7 [b] - 20.5 [a]	840 – 21800	4100 [b] – 4920 [a]	1.29 - 1.31
Tapioca	20.0 [a]	580 – 22400	2660 [a]	2.51
Kuzu	20.0 [a]	480 – 12300	1460 [b] – 1540 [a]	2.09
Lily	20.0 [a] - 20.2 [b]	360 – 18900	2300 [b] – 2310 [a]	2.17
Nagaimo Yam	19.9 [a]	800 – 20000	2000 [a]	3.15
Chestnut	19.9 [a]	440 – 14900	1690 [a]	2.38

767 [1]Adapted from: [a] Tester et al., 2004; [b] Jane, 2009

768 [2]Abbreviations: DP=Degree of polymerization (DP_w = by weight, DP_n=by number of monomers)

Table III. Properties of amylose extracts obtained by aqueous leaching and complex formation approaches[1]

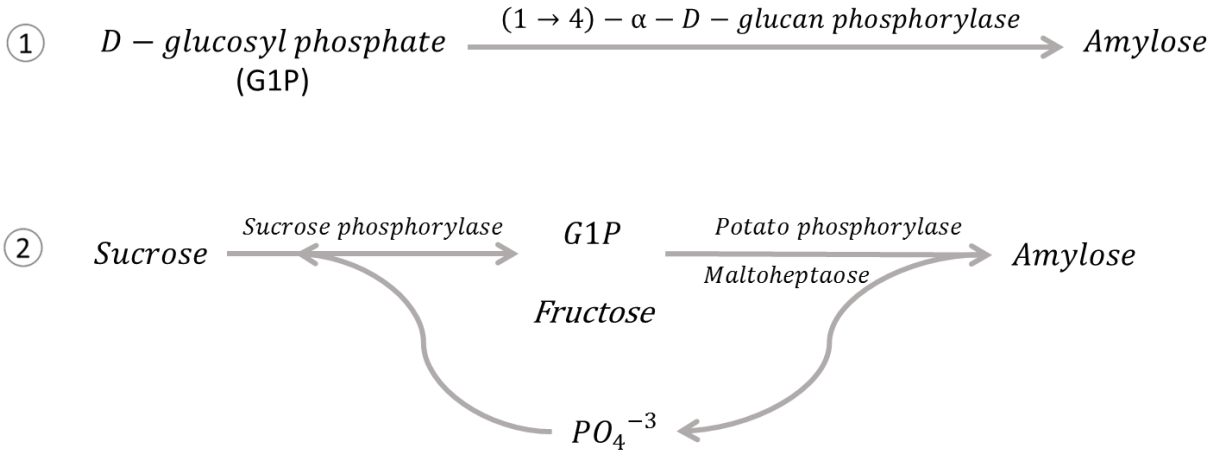
Fractionation method	Treatment	Extract	Reference
Aqueous leaching	Dispersion in dimethyl sulfoxide followed by phase separation Repetitive precipitation with 1-butanol increases purity Heat was needed for complex formation (70°C)	MW = 1.9 X 10^6 IBC = 16.2 IBC (after precipitation) = 18.9	(Killion & Foster, 1960)
	Leaching at 70 - 90°C Annealing of maize and wheat starches	Yield = 22% IBC = 17.6-19.1 DP = 827-1204	(Shi et al., 1991)
Selective precipitation	Dispersion on dimethyl sulfoxide Complexation with 1-butanol	IBC = 19.8 - 20.7	(Adkins & Greenwood, 1969)
	Complexation with 1-butanol	Yield = 23% λmax = 643-655	(Klucinec & Thompson, 1998)
	1-butanol and concanavalin A as precipitating agents	λmax = 608-629	(Corcuera et al., 2007)
	Dispersion in 1-butanol followed by hydrolysis with HCl Ethanol used to wash out 1-butanol	AM Complexes more resistant to acid hydrolysis Narrow MW distribution	(Hu et al., 2013)
	Complexing agent: 1-butanol and iso-pentanol mixture (1:1)	MW = 9 X 10^5 - 5 X 10^6 Positive correlation between MW and degree of hydrolysis	(Naguleswaran et al., 2013)
Combination of methods	Dispersion of high AM starches by using microwaves System fully dispersed	High polydispersity MW = up to 4 X 10^3	(Fishman et al., 1995)
	Leaching at 65-95°C Complexation with 1-butanol after leaching Ultracentrifugation as a step for purification	λmax = 640 IBC = 17.7-20.8 MW = 7.6 X 10^4 - 3.5 X 10^5	(Roger & Colonna, 1996)
	Leaching between 70-80°C for 1h Complexation with 1-butanol after leaching	Polydispersity = 1.36-1.52 DP = 500-1014	(Mua & Jackson, 1997b)
	Dispersion by pressure cooking (140-160°C) Complete dispersion of starch	MW= 1.6-2.5 X 10^5	(Vorwerg et al., 2002)

[1]Abreviations: AM=Amylose; AP=Amylopectin; MW=Molecular weight; IBC=Iodine binding capacity (g 100 g^{-1}), used as a way to quantify purity of AM as well as lambda maximum (λ_{max}); DP=Degree of polymerization (DP_w = by weight, DP_n=by number of monomers); Polydispersity = DP_w/DP_n

Figure captions

Figure 1. Enzymatic procedure for the synthesis of amylose (AM): *(1)* single enzyme system: AM chains by phosphorylation using glucose-1-phosphate (G1P) as a donor of glucosyl units; *(2)* coupled enzyme system: Synthesis of donor (G1P) from sucrose including a recycling step of phosphate groups after phosphorylation towards AM chains. (Adapted from Waldmann et al., 1986).

Figure 2. Fractionation of starch into amylose (AM) and amylopectin (AP) during a hydrothermal treatment (b to c) by phase separation (d). Aqueous phase is rich in AM while gel phase is rich in AP.



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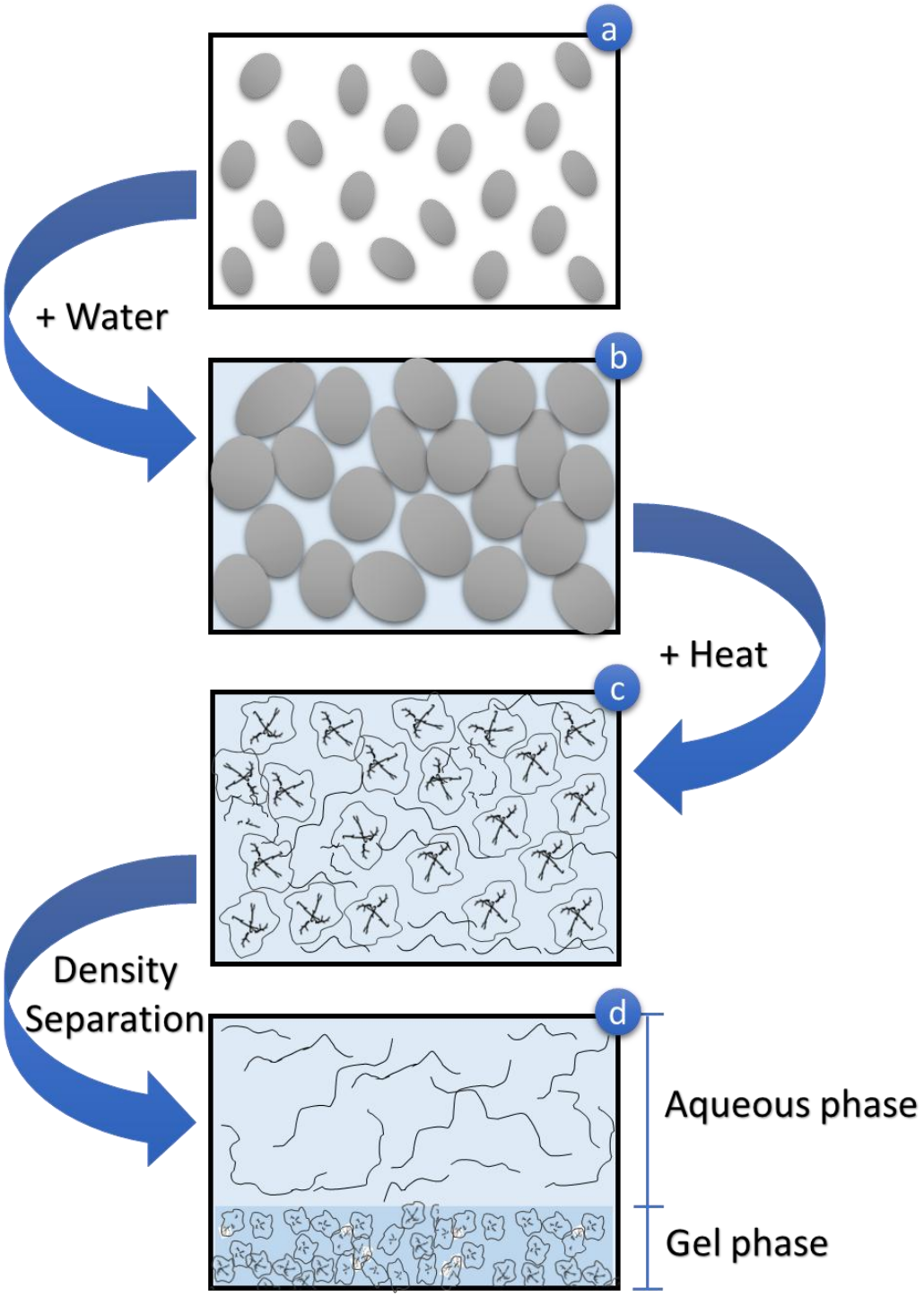

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